Metallothionein and glutathione in *Lymnaea stagnalis* determine the specificity of responses on the effects of ionising radiation

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Abstract. The aim of our study was to distinguish stress-related molecular responses of pulmonate mollusk *Lymnaea stagnalis* from Chernobyl area in comparison with the consequences of other harmful effects. Specimens from feral populations (groups R, T and C, from pond near the Chernobyl nuclear power plant; cooling chanel of hydroelectric power plant and soil-reclamation chanal correspondingly), and specimens adapted to laboratory conditions (control group (CL), disposable exposed to 2 mGy X-ray radiation over the body (RL), exposed to 25° C during 4 days (TL)) were compared. Extremely low level of metallothioneins (MT) and high level of glutathione (GSH) in the digestive gland distinguished group R. In general, Principle Component Analysis distinctly separated laboratory and field groups demonstrating some differences within each set. In the feral groups, low levels of lactate dehydrogenase, MT and protein carbonyls were indicated. Groups T and TL were distinguished by high antioxidative defense and cholinesterase activities. Total superoxide dismutase activities were similar in all groups. The main distinguishing criteria selected by classification and regression tree analysis were the concentrations of protein carbonyls and MT.

1. INTRODUCTION.

Utilisation of molecular biomarkers is fairly accepted to be the most adequate approach for early diagnostic of environmental impacts [1]. However lack of detailed knowledge on their variability limits their use. Furthermore, genetic or adaptive differences between populations complicate the interpretation of studies on different sites. Existing experience in radiobiology is devoted more to the accumulation of radioactive particles in the organism than to the biomarkers of environmental sources of radiation [2, 3]. The monitoring of radioactive contamination associated with Chernobyl disaster with using of molluscs based on the measurement of radionuclide in their shells [4]. Explored biomarkers of received doses are usually connected to the genotoxicity [5]. The attempts to elucidate the biological effects of radioactive discharges in the natural environment by study of other biomarkers, including stress-related proteins, were ineffective [2, 6, 7]. At the same time, it is known that low doses

of γ-irradiation in laboratory conditions induced significant upregulation of the stress-related genes in the aquatic animals [8, 9].

Lymnaea stagnalis (Linné, 1758) a secondary-water lung pond mollusk, can be a suitable object for toxicological study. It belongs to dominant in freshwater zoobentos species on the territory of moderate area of Eurasia. It is known to inhabit most polluted biotopes and accumulate pollution from the water and sediments [10, 11]. However its molecular stress-related responses to inappropriate effects are scant studied [12, 13]. A key goal of this study was to distinguish stress-related molecular responses of pulmonate mollusk *Lymnaea stagnalis* from area of Chernobyl Nuclear Power station (ChAS) in comparison with the consequences of other harmful effects. The set of markers included characteristics of stress and exposure to certain kinds of pollution. Markers of oxidative stress included the activity of the antioxidative enzymes, concentration and redox state of glutathione (GSH), level of oxidative destruction. Metallothioneins (MT), metal-keeping and stress-related proteins, and cholinesterase (ChE) activity as a marker of neurotoxicity [1] also were determined. Finally, to indicate the general and particular signs of responses, integrative statistical data processing was developed.

2. MATERIALS AND METHODS

The experiments were carried out during the August of 2010 year. Adult pulmonate mollusk *Lymnaea stagnalis* with shell height of 25 – 35 mm were caught in three aquatic bodies in Republic of Belarus: from pond Perstok (51°30′ N, 30°01′ E) 14 km from ChAS (group R), heat effluent channel of hydroelectric power plant (group T), and sludgy soil-reclamation chanal near this plant (group C) (sity of Biloozersk, – 52°27′ N, 25°10′ E). Snails from the pond in a clean forestry site located in the upstream portion of river Seret (Ternopil region, Ukraine, 49°49′ N, 25°23′ E) were adapted to laboratory conditions for a 7 days. After that they were divided on three groups, control group (CL), and two groups subjected to effects of radiation or elevated temperature (group RL and TL correspondingly). The snails from the group RL were given 2 mGy X-ray radiation over the body by apparat RUM-20 in a dose 2 m Sv (40 kV, 80 mA, filters 0.5 mm Cu and 0.5 mm Zn, focal length from apparat 40 cm, dosage rate 1 $P \cdot s.^{-1}$, time of exposure 2 s) in a waterless medium and were studied in a seven days. Group TL was exposed to 25° C during 4 days before study. For CL and RL groups, the temperature of water was 18±0.5° C. The mortality of snails was 40% in group RL and 75% in group TL. High mortality stipulated a periods of incubation of treated snails.

For each biochemical parameter eight digestive gland samples were prepared individually. The protein concentration in the supernatant was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Each procedure of tissue analysis was carried out at a temperature around 4 °C.

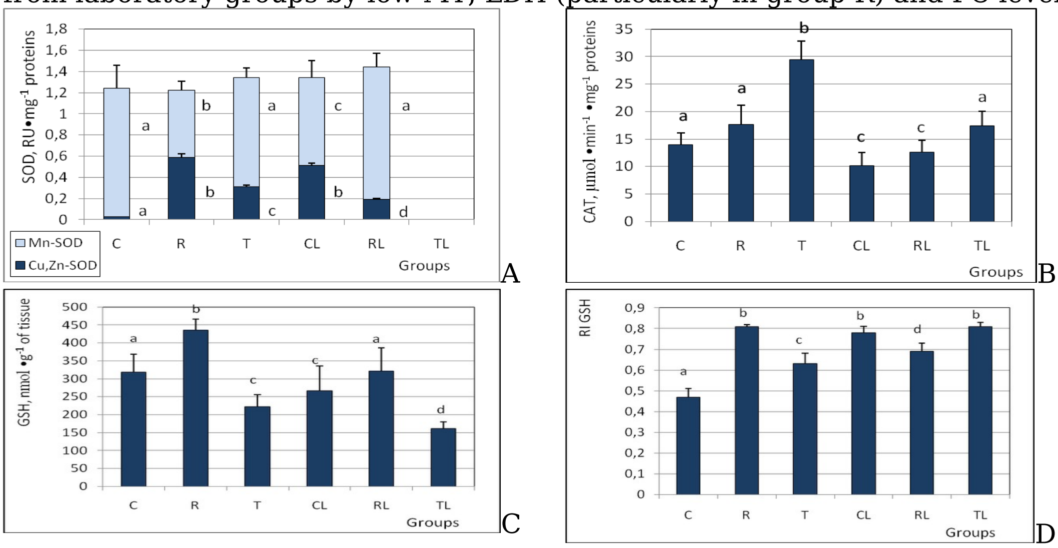
Chemicals: 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), reduced glutathione (GSH), glutathione reductase, 2-vinylpyridine, 1-chloro-2,4-dinitrobenzene (CDNB) nitroblue tetrazolium (NBT), 2,4-dinitrophenylhydrazine (DNPH), serum albumin, phenazine methosulfate, phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, NADH, NADPH, EDTA, acetylthiocholine iodide (ATCh), were purchased from Sigma. All other chemicals were of analytical grade.

The methods applied in this study are described in [14]. Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured according to the method of Beauchamp and Fridovich (1971). In order to assess Mn-SOD activity, the

supernatant was preincubated for 60 min at 0 °C in the presence of 5 mM KCN, which produced total inhibition of Cu,Zn-SOD. Catalase (CAT, EC 1.11.1.6) activity was measured by monitoring the decomposition of 10 mM H_2O_2 according to Aebi (1974) at 240 nm. Glutathione-S-transferase (GST, EC 2.5.1.18) activity was measured according to Habig et al. (1974), using CDNB as substrate. Total glutathione concentration was quantified by the glutathione reductase recycling assay (Anderson, 1985). To estimate the oxidized glutathione (GSSG) level, the protein free sample was treated with 2-vinylpyridine prior to assay run (Griffith, 1980). The redox–index of glutathione (RI GSH) as the ratio of concentrations ([Total glutathione]–[GSSG])/[Total glutathione] was calculated. Lipid peroxidation (LPO) was determined by the production of TBA-reactive substances (TBARS) (Ohkawa et al., 1979). Protein carbonyl (PC) content, as an index of protein oxidation, was measured in the resulting supernatants by the reaction with DNPH (Reznick and Packer, 1994). The activity of lactate dehydrogenase (LDH, EC 1.1.1.27) was determined from the pyruvate-dependent NADH oxidation (Bergmeyer and Bernt, 1974). Cholinesterase (ChE, EC 3.1.1.7) activity as the biochemical marker of neurotoxicity was determined as the ATCh-cleaving ChE activity according to the colorimetric method of Ellman et al. (1961). Metallothioneins (MT) were determined from thiols measure with DTNB according to the method of Viarengo et al. (1997) after the ethanol/chloroform extraction. Results were expressed as means ± standard deviation (SD). Since data were not normally distributed (Lilliefors' test), non-parametric tests (Kruskall–Wallis ANOVA and Mann–Whitney *U*-test) were performed (significant at $p < 0.05$). Data were subjected to principal component analysis (PCA) to evaluate the biomarkers relation both in feral and experimental groups. Classification tree was built using the classification and regression tree (CART) software. All statistical calculations were performed with Statistica v 7.0 and Excel for Windows-2000.

3. RESULTS AND DISCUSSION

Results (Fig. 1) showed that the field groups were distinctly distinguished from laboratory groups by low MT, LDH (particularly in group R) and PC levels.



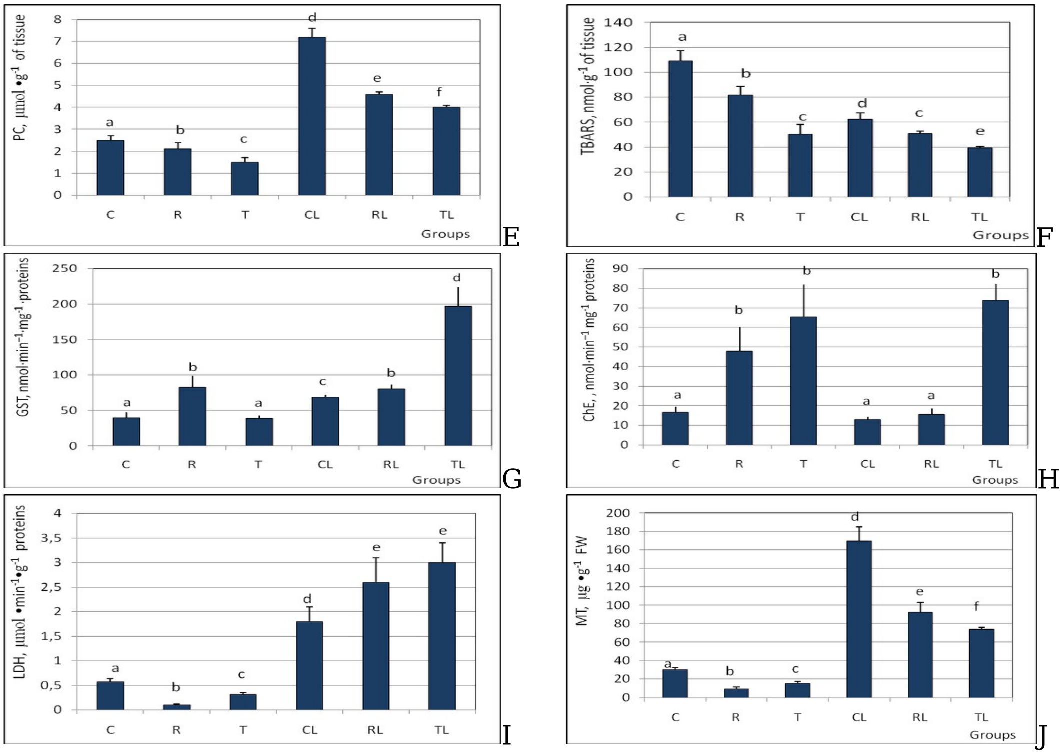
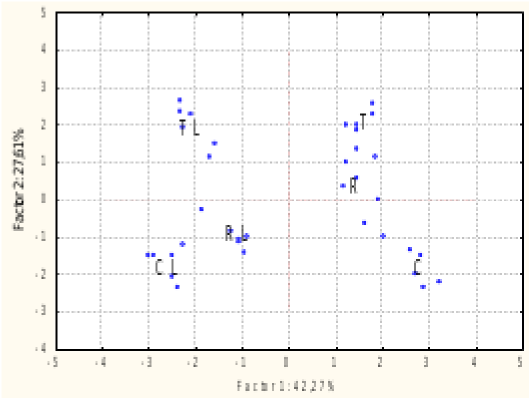


Fig. 1. Biomarkers of stress and toxicity in the digestive gland of *Lymnaea stagnalis.* Data for A, SOD; B, CAT; C, reduced GSH; D, RI GSH; E, PC; F, TBARS; G, GST; H, ChE; I, LDH; J, MT are present as means ± SD (N=8). Same letters correspond to values of biomarkers that are not different significantly, always $P > 0.05$.

In group R, lower MT and highest GSH levels combined with rather high RI GSH and GST activity. To the point, in group RL, the concentrations of MT and GSH showed the same trend compare to CL group. Probably, prolonged existence in the ChAS area provoked depression of the stress-related response based on the expression of MT, which was partly compensated by GSH-dependent functions. Additionally, R group demonstrated comparatively low Mn-SOD activity that may be a consequence of prolong adverse effect of radiation on mitochondria-related processes.

Group C could be qualified as most injured group due to lowest levels of Cu,Zn-SOD, RI GSH, ChE and highest level of TBARS. Groups T and TL were characterized by highest antioxidative activity according to high CAT level and low TBARS and PC levels in correspondent set of groups. However the system of GSH in these snails was low-activity.

A 

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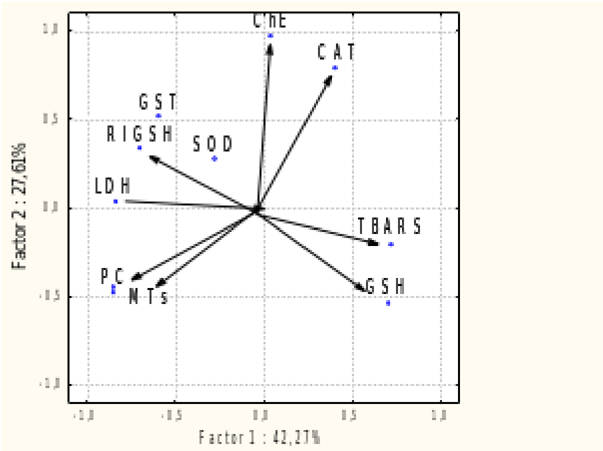

Fig. 2. Centroid grouping (A) and Principal component (B) analysis of *Lymnaea stagnalis* parameters data set in the digestive gland from feral (C, R, T) and laboratory (CL, RL, TL) groups.

According to PCA (Fig. 2), 69.9 % of data belonged to Factors 1 and 2. PCA confirmed differences between field and laboratory groups. One cluster (with the value of p1 less than 0) combined markers that described snails from laboratory. Other set (with the value of p1 more than 0) included groups from field sites. Within each cluster, the groups C and CL, T and TL, R and RL had similar location. At that, R and RL groups situated in the middle of cluster. The similarity of the responses of MT, PC, LDH, RI GSH and GST opposite to the responses of GSH and TBARS (Factor 1) also was confirmed by PCA (Fig. 2B). The responses of ChE and CAT were different from other markers since they belonging Factor 2. To elucidate main partitioning markers, we used the CART algorithm (Fig. 3). When all of the biological parameters at the six groups were compared (Fig. 3A), the PC level in the digestive gland persisted as a splitting variable through two pruning of the tree that resulted in a 6-node tree. MTs and LDH levels in the digestive gland were represented at the nodes as partitioning criteria for the field groups, whilst ChE activity distinguished the laboratory groups, RL and TL. Any of the terminal nodes did not contained misclassified snails. The resulting confusion matrix showed an overall classification accuracy of 93%. The best classification was predicted for group CL, followed by groups TL and RL. Comparison of laboratory group separately demonstrated that the main partitioning criteria were MT and RI GSH levels (Fig. 3B).

Pulmonate mollusc *Lymnaea stagnalis* is haemoglobin-free species [15] that probably makes this snail very sensitive to the oxidative stress. SOD-catalized

reaction can be the source of endogenous oxygen in these circumstances. As it was shown, in general SOD activity remained stable in all studied groups. But the lesser level of Mn-SOD may be a result of particular vulnerability of mitochondrial-dependent processes in the snails from Chernobyl area. The sensitivity of mitochondria to adversive effects also was described in snail *L. stagnalis* [16] and bivalve molluscs [17].

Among studied biomarkers, low weight intracellular thiols, MTs and GSH, that perform crucial biological functions involved in general stress response, particularly scavenging of reactive oxygen species, storage and transport of metal ions [1, 8], were selected as important indexes for the distinguishing of groups (Fig. 3). Upregulation of these stress-related thiols is considered as one of the mechanisms involved in the adaptive response to low dose radiation exposure [8, 9]. The presence of MT in the cells may provide protective effects from radiation-induced genotoxicity and cytotoxicity [8]. However main feature of molluscs from Chernobyl area was the depletion of MT. Probably it was a result of the exhaustion of the stress-induced genes regulation. Despite the unique experience of the radioactive pollution related to the Nuclear Disaster on the ChAS, the data concerning the responses of aquatic animals in the area are scant. Variation in blood cell DNA in *Carassius carassius* from ponds near ChAS determined about ten years after this disaster demonstrated that the abnormalities were not correlated with known contaminant distributions [18]. Genetic studies, by electrophoresis of seven Dreissena polymorpha populations in the water basins near the ChAS, also revealed that the differences in populations apparently governed by conditions at the breeding site and not by thermal or radioactive contamination [6]. So, peculiarities found in the MT concentration deserve attention and further study.

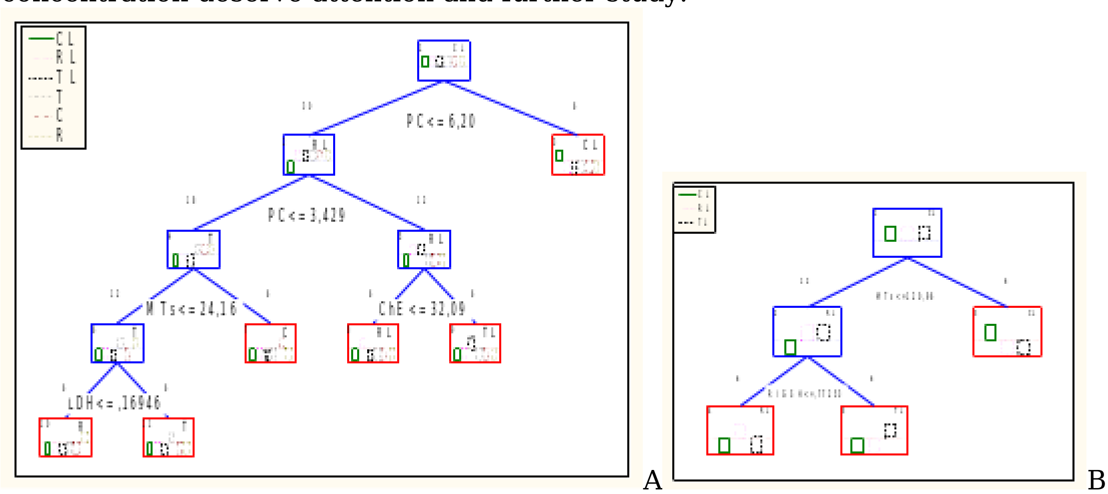

Fig. 3. Classification tree models. Terminal nodes identify the groups and the number of mollusks represented all studied specimens (A) and only laboratory exposured groups (B).

Besides these peculiarities, molluscs from ChAS area had not remarkable differences compare to other field groups. An increase of anaerobic glycolysis is well approved sign of toxic effect in mollusks [19]. In our study, despite the favourable regime of respiration, laboratory groups had comparatively high LDH activity that attests the shift to anaerobiosis. High levels of PC, MT and RI GSH in the laboratory snails could be explained by slow removal of damaged proteins and maintaining of reset state in these conditions. Opposite regularity was correspondent to the field groups. Low level of PC in these snails can be

explained by high turnover of proteins [17]. The enhancement of pulmonary respiration in *L. stagnalis* in these groups has been found to be associated with a rise of levels of reduced glutathione and TBA-reactive products in the tissue [20]. The relation between GSH and TBARS levels was confirmed in our study by PCA.

Thus the prolonged effect of radiation caused particular response of stress-related systems, based probably on the inhibition of the expression of correspondent proteins. On the other hand, elevated temperature provoked the activation of these systems in molluscs, as it is evident from our data and other results [2] and in fish [21].

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